Best Practice Guidelines for sarcoma testing by reverse transcriptase PCR assays

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1. INTRODUCTION AND SCOPE

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and fluorescent in situ hybridization (FISH) are both technologies used for the detection of translocations in a wide variety of cancers including a number of sarcomas. These techniques are complementary and testing laboratories should consider testing using both technologies. Testing laboratories should be aware of the limitations of both techniques and test and report accordingly. FISH testing using breakapart probes do not identify the partner gene in the translocation, therefore a definitive diagnosis cannot be provided. Testing by RT-PCR can identify the partner gene information which can provide relevant diagnostic and prognostic information. Where RT-PCR testing or the use of dual fusion probes is not possible within the same laboratory, arrangements should be made to have this performed at another testing laboratory. Limitations of RT-PCR are discussed in this document.

These recommendations are intended as a reference tool for points to consider when testing sarcoma samples by RT-PCR assays. They should be used within local testing and reporting arrangements (e.g. within a local laboratory and associated clinical departments).

They incorporate the standards required by regulatory bodies Clinical Pathology Accreditation (CPA) UK Ltd/ United Kingdom Accreditation Service and Medical Laboratories – Requirements for quality and competence (ISO 15189:2012) and by statute (Clinical Governance), while taking into account current practice in the U.K.

It must be noted that these recommendations are minimum requirements and that professional judgement is of paramount importance for many circumstances.

The use of 'shall' in this document indicates a requirement and the use of 'should' indicates a recommendation.

Where there appears to be contradiction between available recommendations/guidelines, the most recently published should be taken to apply to all.

All diagnostic laboratories shall be accredited to nationally or internationally accepted standards. Laboratories shall participate in an External Quality Assessment (EQA) for all aspects of their service for which a scheme is available.

2. Disease Overview

2.1 Alveolar rhabdomyosarcoma, t(2;13)(q35;q14)/ t(1;13)(p36;q14)

PAX3 (Paired box gene 3)/ FOXO1 (Forkhead box O1A)
PAX7 (Paired box gene7)/ FOXO1 (Forkhead box O1A)

Rhabdomyosarcoma (RMS) accounts for approximately 3% of cancers in children and 2% in adolescents. Of all subtypes, embryonal rhabdomyosarcoma (ERMS) and alveolar rhabdomyosarcoma (ARMS) are the most common in this age group. ARMS arises most commonly in the vicinity of skeletal muscle in the extremities and trunk. Other sites include the paraspinal and the perineal regions as well as the paranasal sinuses. It occurs primarily in adolescents and young adults with a median age of 6.8 to 9 years. The prognosis for children and adolescents is related to a number of factors including age of patient, site of the primary tumour, presence of metastases at diagnosis and histopathological subtype. Histologic subtype has been incorporated into risk stratification in more recent clinical trials: in the Societe Internationale d'Oncologie Pediatrique (SIOP). ARMS is generally associated with a poorer prognosis than ERMS and accounts for 20% of all RMS cases As there can be overlap between the histological features of both subtypes and the clinical behaviour of ARMS without translocation is more similar to that of the ERMS, detection of the translocation is relevant for the differential diagnosis and prognosis. ERMS is not characterized by a recurrent translocation.

Molecular Profile
The balanced translocations, t(2;13)(q35;q14) or t(1;13)(p36;q14) are consistently and specifically associated with ARMS. These translocations are detected in up to 80% of cases and generate the fusion genes *PAX3–FOXO1* or *PAX7–FOXO1*. These fusion genes can be detected using commercially available fluorescence in-situ hybridisation (FISH) probes (fusion probes or indirectly using break-apart probes) or RT-PCR.

Table 1 – Fusion transcripts found in ARMS

Reference Sequence	Gene	Exon (after which breakpoint occurs)	Reference Sequence	Gene	Exon (before which breakpoint occurs)	Frequency
NM_181457.3	*PAX3*	7	NM_002015.3	*FOXO1*	2	60%
NM_001135254.1	*PAX7*	7	NM_002015.3	*FOXO1*	2	20%

2.2 Desmoplastic Small Round Cell Tumour, t(11;22)(p13;q12.2)

EWSR1 (Ewing Sarcoma Breakpoint 1)/ WT1 (Wilms' Tumour suppressor gene)

Desmoplastic small round cell tumour (DSRCT) is a sarcoma usually seen in children and young adults, typically males between the ages of 15 and 35 years. It usually presents as a large mass in the abdomen or pelvis or more rarely in the pleura, thorax and central nervous system. It comprises of nests of small round cells embedded in a fibrous (desmoplastic) stroma. The immunoprofile shows some overlap with other round cell tumours: CD99 is expressed in up to one third of these tumours. Characteristically there is dot-like expression of desmin and the majority of the tumours co-express EMA and cytokeratins. Myogenin and MyoD1 are negative. Overall survival is poor despite multi-modality therapy and this is primarily due to local disease and metastatic spread.

Molecular Profile

DSRCT is characterised in almost all cases by a t(11;22)(p13;q12.2) translocation leading to an oncogenic *EWSR1-WT1* gene fusion. There is heterogeneity within the *EWSR1-WT1* gene fusion, with several splice variants having been described, however all encode an aberrant transcriptional regulatory factor comprised of the NH_2 terminal effector region of *EWSR1* and the COOH terminal region of *WT1* leading to DSRCT progression [1]. Rare cases diagnosed as DSRCT containing a *EWSR1-ERG* and *EWSR1-FLI1* translocation have been described, however the exact nature of these cases remain controversial.

Table 2 – Fusion transcripts found in DSCRT

Reference Sequence	Gene	Exon (after which breakpoint occurs)	Reference Sequence	Gene	Exon (before which breakpoint occurs)	Frequency
NM_005243.3	*EWSR1*	7	NM_024426.4	*WT1*	8	85-90%
NM_005243.3	*EWSR1*	8	NM_024426.4	*WT1*	8	<15%
NM_005243.3	*EWSR1*	9	NM_024426.4	*WT1*	8	
NM_005243.3	*EWSR1*	5	NM_024426.4	*WT1*	10	

2.3 Infantile Fibrosarcoma, t(12;15)(p13.2;q25.3)

ETV6 (ETS Variant Gene 6)/ NTRK3 (Neurotrophic Tyrosine Kinase receptor type 3)

Infantile fibrosarcoma classically presents as a solitary rapid enlarging tumour in the first year of life, 36-80% being congenital [2]. These are usually densely cellular tumours that, in areas, resemble the adult-type counterpart, with the cells arranged in herringbone pattern. Immunohistochemistry is usually not helpful in reaching the diagnosis. Local recurrence may occur however infantile fibrosarcoma rarely metastasizes. Unlike the more aggressive adult-type fibrosarcoma, infantile fibrosarcoma has a favourable prognosis with a survival rate of 90%.

Molecular Profile

Infantile fibrosarcoma is characterised by a reciprocal t(12;15)(p13.2;q25.3) translocation leading to an oncogenic *ETV6-NTRK3* gene fusion. The HLH domain of *ETV6* is fused with the protein tyrosine kinase domain in *NTRK3*, dimerization of the chimeric protein mediated by *ETV6*, causes activation of the protein tyrosine kinase domain in *NTRK3* leading to dysregulation of *NTRK3* signal transduction pathways [3]. The *ETV6-NTRK3* gene fusion is also present in the histologically similar congenital mesoblastic nephroma [4], as well as in acute myeloblastic leukaemia [5] and breast secretory carcinoma [3]. The translocation is not present in infantile fibromatosis, adult-type fibrosarcoma, or other soft tissue sarcomas [1].

Table 3 – Fusion transcripts found in Infantile fibrosarcoma

Reference Sequence	Gene	Exon (after which breakpoint occurs)	Reference Sequence	Gene	Exon (before which breakpoint occurs)	Frequency
NM_001987.4	*ETV6*	5	NM_002530.3	*NTRK3*	15	70%

2.4 Synovial Sarcoma, t(X;18)(p11.2;q11.2-11.23)

SS18 (Synovial Sarcoma Translocation, Chromosome 18)/ SSX1 (Sarcoma Synovial X Breakpoint 1)
SS18 (Synovial Sarcoma Translocation, Chromosome 18)/ SSX2 (Sarcoma Synovial X Breakpoint 2)
SS18 (Synovial Sarcoma Translocation, Chromosome 18)/ SSX4 (Sarcoma Synovial X Breakpoint 4)

Synovial sarcomas (SS) can present in patients of all ages, although more commonly occurring in young adults aged 20 to 40 years. They usually present as a deep-seated tumours more frequently in the lower or upper extremities, adjacent to the joints. However, 15% may arise in the trunk and 5% in the head and neck region [2]. Up to one third of the cases show calcification/ossification and this can be detected by imaging. Histological subtypes include monophasic fibrous, biphasic and poorly differentiated. Generally, some degree of epithelial differentiation can be detected by immunohistochemistry (EMA and cytokeratins). Synovial sarcomas can also express TLE-1, CD99, bcl-2 and S100, the latter usually showing focal expression.

SS are high grade tumours with generally poor outcomes in the long term. Prognosis depends on several features including size, age, completeness of initial surgical procedure and percentage of poorly differentiated areas. The majority of metastasis is to the lung, and less frequently to bone.

Molecular Profile
Synovial sarcoma is characterized by a specific t(X;18)(p11.2;q11.2-11.23) translocation, which leads to a fusion between the transcriptional activation domain of *SS18* and the repressor domain of the highly homologous *SSX1, SSX2*, or *SSX4* genes. Tumorigenesis is thought to be via transcriptional dysregulation of specific target genes.*SS18-SSX* fusion variants of all three translocations are rare and have only been found in isolated cases. The co-existence of *SS18-SSX1* and *SS18-SSX2* translocations is reported to be found in approximately 10% of cases, and up to 5% of cases have no associated translocations.

Table 4 – Fusion transcripts found in synovial sarcoma

Reference Sequence	Gene	Codon (after which break-point occurs)	Reference Sequence	Gene	Codon (before which break-point occurs)	Frequency
NM_005637.2	*SS18*	410	NM_005635.3	*SSX1*	111	64%
NM_005637.2	*SS18*	410	NM_003147.5	*SSX2*	111	35%
NM_005637.2	*SS18*	410	NM_005636.3	*SSX4*	111	1%

2.5 Ewing's Sarcoma, t(11;22)(q24;q12)

EWSR1 (Ewing Sarcoma Breakpoint 1)/ FLI1 (Friend Leukemia Virus Integration 1)
EWSR1 (Ewing Sarcoma Breakpoint 1)/ ERG (V-ETS Avian Erythroblast virus E26 oncogene)
EWSR1 (Ewing Sarcoma Breakpoint 1)/ ETV1 (ETS Variant Gene1)
EWSR1 (Ewing Sarcoma Breakpoint 1)/ FEV (Fifth Ewings Variant)
FUS (Fused in Sarcoma)/ ERG (V-ETS Avian Erythroblast virus E26 oncogene)

The Ewing's sarcoma family of tumours (including Ewing's sarcoma, Askin tumour and peripheral primitive neuroectodermal tumour) comprises highly malignant small round cell tumour of bone and soft tissue (OMIM). It is the second most common primary bone malignancy after osteosarcoma in Europe. It mainly affects children and adolescents, has a median age of onset of 15 years of age and affects more males than females. The majority of Ewing's sarcomas arise from the medullary cavity of long bones, pelvis, ribs, vertebrae, mandible and clavicle and approximately 25% of cases occur in soft (extraosseous) tissue. Metastases are present at diagnosis in approximately 25% of cases and commonly occur in the lung, bone and bone marrow. Histologically, sheets of relatively uniform small round cells with indistinct cytoplasmic borders are seen. Necrosis is frequently present and viable cells are seen around blood vessels. Classically Ewing's sarcomas display membranous type CD99 expression. Cytokeratins are also expressed in up to 30% of cases [5].

Treatment includes a combination of surgery, chemotherapy and radiation and the prognosis has improved over the years such that now two thirds of patients are cured of their disease[2]. However for patients with metastatic disease the outcome is poor. [1,6]

Molecular Profile

Ewing's sarcoma are characterised by specific acquired chromosome translocations which result in gene fusions that are shown in table 5. Approximately 85% of cases show an *EWS-FLI1* gene fusion where the DNA binding domain of the ETS transcript is fused to the transactivation domain of the ETS transcription factor *FLI1* generating an oncogenic chimeric fusion protein. Approximately 60% of cases with *EWS-FLI1* fusions are type 1, where exons 1-6 of *EWS* are fused to exons 6-9 of *FLI1*. The remaining cases have type 2 fusions where exons 1-7 of *EWS* are fused to exons 5-9 of *FLI1*. Approximately 10% of cases are caused by fusion of *EWS* and *ERG*, which is also a member of the ETS family of transcription factors and is closely related to *FLI1*.

Table 5 – Fusion transcripts found in Ewing's Sarcomas

Reference Sequence	Gene	Exon (after which break-point occurs)	Reference Sequence	Gene	Exon (before which break-point occurs)	Frequency
NM_005243.3	*EWSR1*	6 7	NM_002017.4	*FLI1**	6-9 5-9	60% 25%
NM_005243.3	*EWSR1*	7	NM_004449.4	*ERG*	6	10%
NM_005243.3	*EWSR1*	7	NM_004956.4	*ETV1*	10/11	<1%
NM_005243.3	*EWSR1*	7	NM_017521.2	*FEV*	2	<1%
NM_004960.3	*FUS*	1-6	NM_004449.4	*ERG*	9	<1%

2.6 Clear cell Sarcoma of soft tissue, t(12;22)(q13;q12)

EWSR1 (Ewing Sarcoma Breakpoint 1)/ ATF1 (Activating Transcription Factor 1)

Clear cell sarcomas are tumours that affect teenagers and young adults and are found in the deep soft tissues of the lower limbs close to tendons and aponeuroses. The foot/ankle region is the most common site, affected in 40% of the cases [2]. Histologically, these tumours feature spindle to epithelioid cells with a characteristic nested growth pattern. Scattered multinucleated giant cells are usually present. Clear cell sarcomas express melanocytic markers (HMB45 and Melan A) and S100. Melanin pigment is usually not seen. Local recurrence is common and metastases occur in the lymph nodes, lung, liver brain and bone. The 5 year survival rate is estimated to be 40-67%.

A similar tumour-type can be found in the gastro-intestinal tract. In spite of having similar morphology and molecular profile, at this site, there is no expression of melanocytic markers and therefore is considered to

be a different disease and referred to by some authors as clear cell sarcoma-like tumours of the gastrointestinal tract [4]. In addition, the same translocation has been identified in clear cell salivary gland carcinomas with myoepithelial differentiation [7].

Molecular Profile
Clear cell sarcoma is characterised by a specific acquired chromosome translocation *EWS-ATF1*. A chimeric oncogenic protein is generated when the N-terminal domain of EWS is fused to the basic leucine zipper (bZIP) domain of the activating Transcription factor 1 protein.

Table 6 – Fusion transcripts found in Clear cell sarcoma

Reference Sequence	Gene	Exon (after which breakpoint occurs)	Reference Sequence	Gene	Exon (before which breakpoint occurs)	Frequency
NM_005243.3	*EWSR1*	8	NM_005171.4	*ATF1*	5	>90%

2. 7 Myxoid Liposarcoma, t(12;16)(q13;p11)

FUS (Fused in Sarcoma/ DDIT3 (DNA Damage inducible transcript 3)
EWSR1 (Ewing Sarcoma Breakpoint 1)/ DDIT3 (DNA Damage inducible transcript 3)

Myxoid Liposarcomas are soft tissue tumours that can affect any age group with a peak incidence between 35 and 55 years of age. Both genders are equally affected. These sarcomas are frequently sited within the musculature of the thigh (2/3 of the cases). Metastases to the lung and unusual extrapulmonary sites including the serosal surfaces, axilla and contra-lateral limb can occur. Histologically they are multinodular tumours comprising stellate to round cells embedded in a highly vascular myxoid stroma. The vasculature is characteristic and comprising delicate arborizing blood vessels ('chicken-wire'). Lipoblasts are frequently seen around these vessels. A cellular round cell component can be seen and is related to poorer prognosis (myxoid/round cell liposarcoma). Immunohistochemistry is not helpful in diagnosing the tumour.

Molecular Profile
Myxoid liposarcoma has a specific acquired chromosome translocation in which Chimeric oncogenic proteins are generated when the N-terminal transactivation domain of the RNA binding protein Fusion in Sarcoma (*FUS*) transcript (or rarely *EWSR1*) is fused to the DNA binding and leucine zipper dimerisation domain of the transcription factor CCATT enhancer binding protein (*DDIT3*).

Table 7 – Fusion transcripts found in Mixoid liposarcomas

Reference Sequence	Gene	Exon (after which break-point occurs)	Reference Sequence	Gene	Exon (before which break-point occurs)	Frequency
NM_001195053.1	*DDIT3*	4	NM_004960.3	*FUS*	5	95%
NM_001195053.1	*DDIT3*	4	NM_005243.3	*EWSR1*	2-3	Rare

2.8 Low Grade Fibromyxoid Sarcoma, t(7;16)(q33;p11).

FUS (Fused in Sarcoma)/ CREB3L2 (cAMP Response Element-Binding Protein)/ CREB3L1

Low grade fibromyxoid sarcoma usually presents as a well-defined myofibroblastic tumour characterized by spindle cells with wavy cytoplasm arranged in moderately cellular fascicles or storiform growth patterns in collagenized and myxoid zones. MUC4 antibody has been proven very helpful in aiding the diagnosis of this tumour type, being both sensitive and specific amongst the spindle cell tumours. Patients of any age or

gender can be affected but mainly young adults and up to 20% of cases are in patients below the age of 18 years.

Molecular Profile
These tumours are characterised by a recurrent translocation t(7;16)(q33;p11). This is the only cytogenetic abnormality in approximately two thirds of cases. Approximately 25% of cases demonstrate a ring chromosome. These abnormalities result in the fusion of the 5' part of *FUS* gene on 16p11 with the 3' part of the *CREB3L2* gene on 7q33. Molecular studies identified these as *FUS-CREB3L2* and *FUS-CREB3L1* in 75-96% and 4-6% of cases respectively. The chimeric *FUS-CREB3L2* fusion gene functions as an aberrant transcription factor which causes the deregulated expression of wild type *CREB3L2* target genes.

Table 8 – Fusion transcripts found in Low Grade Fibromyxoid Sarcoma

Reference Sequence	Gene	Exon (after which break-point occurs)	Reference Sequence	Gene	Exon (before which breakpoint occurs)	Frequency
NM_004960.3	*FUS*	5-7	NM_194071.3	*CREB3L2*	5	76-96%
NM_004960.3	*FUS*	7-9	NM_052854.1	*CREB3L1*	5	Rare

2.9 Extraskeletal Myxoid Chondrosarcoma, t(9;22)(q22;q12)

NR4A3 (Nuclear Receptor Subfamily 4)/ EWSR1 (Ewing Sarcoma Breakpoint 1)
NR4A3 (Nuclear Receptor Subfamily 4)/ TAF15 (RNA Polymerase II)

Extraskeletal myxoid chondrosarcoma (ESMC) are tumours of uncertain differentiation that usually occur in adults, twice as frequent in male patients, in the deep soft tissues of the proximal extremities. However, they can be found anywhere in the body. These tumours have a multinodular pattern of growth featuring delicate spindle or round cells that tend to interconnect, forming cords in areas. Real cartilaginous differentiation is not seen. A small subset of these tumours tend to be more cellular and display rhabdoid morphology. ESMC has high rates of local recurrences and metastatis.

Molecular Profile
Extraskeletal Myxoid Chondrosarcomas are characterised by a t(9;22)(q22;q12) translocation or less frequently a t(9;17)(q22;q11) or t(9;15)(q22;q21) translocation. The translocation is the fusion between the *NR4A3* gene on 9q22 and the *EWSR1* on 22q12 or *TAF15* on 17q1. The *TAF15* gene alternatively known as *CHN* or *NOR1* encodes an orphan nuclear receptor which belongs to the steroid/thyroid receptor gene. The *EWSR1* and *TAF15* genes belong to the TET family of genes.

Table 9 – Fusion transcripts found in Extraskeletal Myxoid Chondrosarcoma

Reference Sequence	Gene	Exon (after which break-point occurs)	Reference Sequence	Gene	Exon (before which breakpoint occurs)	Frequency
NM_173200.2	*NR4A3*	3	NM_005243.3	*EWSR1*	12	64%
NM_173200.2	*NR4A3*	3	NM_139215.2	*TAF15*	6	27%

3. METHODOLOGY

3.1 Preparation of material prior to receipt in testing laboratory

The testing laboratory should be familiar with the pre-analytical protocols used in the handling of specimens prior to testing. In particular, centres used for the referral of clinical samples from multiple sources shall endeavour to identify as much as possible about pre-analytical conditions and protocols. It may not be

possible to alter these conditions but in the event of inadequate complementary DNA (cDNA) quality or where it has the potential to compromise testing in any way, such conditions shall be identified and cited in the final report.

Formalin is the standard fixative for pathology samples. A solution to 10% formalin strength (4% formaldehyde in solution) shall be used. Formalin fixatives such as 10% buffered or un-buffered formalin may be mixed with saline. Formalin-fixed paraffin-embedded (FFPE) material should be used for sarcoma translocation testing.

Formalin fixation is a time-dependent chemical reaction [8] and as such delay in fixation or too long a time in fixative can inhibit subsequent analysis [9]. The tissue shall therefore, be fixed to an optimal level with consideration taken for size and consistency of the sample.

Fixatives that are not formalin-based shall be tested under a fully validated procedure prior to routine use.

It may be necessary to perform decalcification prior to microtomy. Formic acid or EDTA are standard solutions used for this purpose and shall be used for an appropriate period in a controlled manner. If decalcification for longer than 12 hours is necessary, checks to ensure decalcification shall be performed to maintain the integrity of the sample. Strong, mineral acids such as hydrochloric acid shall not be used for this purpose [10].

3.2 Sample Requirements

The neoplastic cell content of each sample shall be estimated prior to analysis. Sectioning of FFPE material shall be performed. These should be either mounted on glass slides or prepared as 'curls' into appropriate tubes. A section stained with a suitable morphological method such as Haematoxylin & Eosin shall be cut after the test sections have been prepared and used for the estimation of neoplastic cell content. This shall be referred to in the final report.

Where the neoplastic cell content is not known then this shall be referred to in the final report.

Fresh tissue and infiltrated bone marrow can be a source of neoplastic cells for testing and maybe considered.

3.3 RNA extraction

The laboratory shall have procedures in place to avoid contamination. PCR products shall not be brought in to clean areas where nucleic acid extraction and PCR set up is performed. The laboratory shall have suitable procedures in place to ensure that any tube transfer step maintains the correct order of tubes.

A number of different methodologies, both manual and automated, are available for the reliable extraction of ribonucleic acid (RNA) from FFPE material. The method of choice is dependent upon the testing laboratory.

3.4 Quantification of RNA

The extracted RNA shall be quantified. There are a number of suitable methodologies for accurately quantifying the concentration of RNA. Methods which incorporate a dye into the nucleic acid such as Qubit® will give a more accurate quantification, however these kits are costly. The Nanodrop® which measures the optical density of the DNA at 260nm is adequate, providing a DNase step has been incorporated into the RNA extraction protocol, since the Nanodrop® is unable to distinguish between single and double stranded nucleic acids.

3.5 Preparation of cDNA

Following RNA extraction it is necessary to synthesise complementary DNA (cDNA). Commonly used kits utilise random hexamer primers and either Superscript III enzyme (Invitrogen) or High Capacity cDNA RT kit (Life Technologies) or Moloney murine leukaemia virus reverse transcriptase (MMLV-RT). All kits, in experienced hands, yield sufficient quality of cDNA for testing, providing the RNA used is not too degraded.

A no template control (NTC) shall be included with each reverse transcriptase run.

Gene specific primers may be used. These have the advantage of being highly specific however when multiple genes require to be analysed each specific translocation will require different cDNA to be synthesised. In this instance there may be insufficient RNA to perform all the investigations required.

3.6 Housekeeping genes

The integrity of RNA extracted from FFPE tissues and its amplification by PCR is affected by a number of factors such as length of storage before analysis, fixation time, fixation type and thickness of the tissue [11]. The quality of the cDNA for amplification shall be assessed using a reference housekeeping genes. *HMBS and HPRT1* are stable housekeeping genes for (q)RT-PCR applications in cells or tissues of mesenchymal origin [12,13] such as soft tissue sarcomas. Other commonly used suitable genes are *BBDG*, *β actin* and *G6PD.*

The PCR products of the housekeeping genes shall be in the size range of the specific target amplicons for the different translocations.

The primer sequences for a number of recommended housekeeping genes is presented in Appendix 1.

3.7 Primer sequences

The design of the primers is crucial for identifying the specific transcript of interest. This is often a limitation of performing RT-PCR.

The primer sequences for a number of recommended amplicons are presented in Appendix 2.

3.8 Controls

Suitable controls of comparable material to the test material shall be included in every assay. Samples of known status for sarcoma translocation shall be used as controls. Such controls shall be derived from FFPE material. Apart from clinical samples, these may be characterised cell lines fixed in the same fixative and processed as cell pellets to paraffin wax under conditions comparable with routine samples [8]. Where clinical samples are used these shall be validated either by a different laboratory, or confirmed by Sanger sequencing.

The validity of RT-PCR results as evidence of specific gene expression depends on the use of appropriate controls. Negative controls are designed to demonstrate absence of PCR products in amplifications of cDNA from cells that do not express the gene as well as reactions that do not contain template (NTC).

Positive controls shall provide evidence that the primer set has worked and that the reaction has been set up correctly. Therefore the positive control should contain the same reagents used in the case tested plus a template that is certain to contain the gene tested [14]. This is particularly important in the case of a negative result.

4. RESULTS AND REPORTING

4.1 Analysis of results

All results shall be checked and confirmed independently by a suitably trained and qualified technician, scientist or pathologist. This check shall be documented.

All controls included in the test shall be checked. An assay shall be interpreted as a fail if any of the controls fail to yield the expected result.

4.2 Confirmation of results

Confirmation of results by duplicate analysis shall be performed. This can be achieved by numerous protocols and depends upon local practice e.g. duplicate RNA extractions and subsequent testing, duplicate cDNA reactions on the same RNA extracted sample, duplicate RT-PCRs on the same cDNA sample, or subsequent repeat reactions after first round of testing.

If unusual or unexpected results are obtained e.g. a different sized PCR product as expected, then the sample shall be analysed further by other techniques to validate the results.

4.3 Reporting of results

It is acknowledged that reporting formats vary between laboratories according to type of laboratory, local information technology (IT) systems and local reporting arrangements such as integrated reporting with other test results.

The final reports shall include the following details:

- Two unique patient identifiers
- Sample identifier
- A clear overall result (e.g. in bold or large text).
- Details of which tests have been performed
- Methodology of tests performed
- Statement regarding the amplification of the housekeeping gene(s) including which gene, the size of the product(s), and the quality of result as well an interpretation of this result in the context of the size range of the specific translocation that is tested for. The interpretation can be sufficient or insufficient for this test. If the quality of the result is insufficient, the specific RT-PCRs for the translocations cannot be evaluated.
- Specific fusion transcript tested
- If method(s) distinguish between different translocations then the breakpoints shall be stated.
- If breakpoints are described using exon numbering then an appropriate reference sequence including version number shall be provided.

It is good practice to provide the reader of the report with all relevant information in order to interpret the results appropriately. Therefore the reports should include the following details:

- The limitations of the test(s) performed i.e. poor RNA quality or poor starting sample quality
- In certain cases where different types of fusion transcripts may be identified (i.e. *EWS-FLI1* type I or type II), the reference to the specific type must be included in the report.

It is not recommended to use the terms positive/negative for the absence or presence of a particular mutation/translocation as these terms can be easily misinterpreted. It is recommended to use the terms ‘detected’/’not detected’ or ‘present’/’absent’.

Where the neoplastic cell content is not provided or is not known, then it is acceptable to report the result provided that the limitations of the test performed are clearly stated. It is recommended to use the term ‘no evidence of…’.

4.4 Interpretation of results

All reports shall include clear and concise interpretation of the results.

When a translocation has been detected then the report shall state that the results either ‘confirms the diagnosis or ‘supports the diagnosis of XX’.

When a translocation has not been detected then the report shall state that the results neither confirms nor excludes the diagnosis of ‘XX or “does not support the diagnosis of XX nor fully excludes the diagnosis of XX because a small percentage of these sarcomas do not have this translocation.

The report shall state that the final result should be interpreted in the light of histological, clinical and radiological context. All sarcoma cases shall be discussed at a relevant multidisciplinary team (MDT) meeting for a clinical decision.

It is recognised that many molecular diagnostic laboratories integrate sarcoma RT-PCR testing results with other molecular test results, immunohistochemistry and histopathological features. The laboratory should ensure that the sarcoma RT-PCR test result is not mis-reported or open to mis-interpretation when transposed into an integrated report.

5 SOURCES

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6 APPENDICES

Appendix 1: Primer sequences for suitable Housekeeping genes

Gene	Primer name	Primer sequence (5'-3')	Reference
G6PD	86 F	ACGGCAACAGATACAAGAAC	15
	141F	CCAAGAAGCCGGGCATGT	
	200 F	GCGCAACGAGCTGGTGAT	
	G6PD R	CGAAGTGCATCTGGCTC	
Beta 2 microglobulin	B2M-246 F	TGA CTT TGT CAC AGC CCA AGA TA	16
	B2M-230 R	AAT CCA AAT GCG GCA TCT TC	
	B2M-P	VIC-TGA TGC TGC TTA CAT GTC TCG ATC CCA	
HMBS	HMBS F 150	TGCCAGAGAAGAGTGTGGTG	Not Available
	HMBS R150	ATGATGGCACTGAACTCCTG	
	HMBS F 250	CTGGTAACGGCAATGCGGCT	
	HMBS R250	TTCTTCTCCAGGGCATGTTC	
	HMBS R665	GTTAAGCTGCCGTGCAACATC	

Appendix 2: Primer sequences for fusion transcripts.

1. Alveolar Rhabdomyosarcoma

Primer name	Primer sequence (5'-3')	Reference
PAX3-SH F	AGACAGCTTTGTGCCTCCGTC	17
PAX7-SH F	GCTTCTCCAGCTACTCTGAC	
FKHR-SH R	TTCTGCACACGAATGAAC	
PAX7 F	GCTTTCCCAGCTACTCTGAC	Not available
PAX3 F	AGACAGCTTTGTGCCTCCGTC	
FKHR R	TTCTGCACACGAATGAAC	
PAX3 F	TTGGCAATGGCCTCTCACC	17
FKHR R	ATCCACCAAGAACTTTTTCCAG	
PAX3/7pr VIC	VIC CCCTACACAGCAAGTTCATTCGTGTGCAG	
PAX7FW	CAACCACATGAACCCGGTC	

2. Ewings Sarcoma

Primer name	Primer sequence (5'-3')	Reference
EWS/FLI F	CCAAGTCAATATAGCCAACAG	18
EWS/FLI R	GGCCAGAATTCATGTTATTGC	
EWS-22.3 F	TCCTACAGCCAAGCTCCAAGTC	
ERG-9 R	ACTCCCCGTTGGTGCCTTCC	
EWS/10-SH F	GGATGAAGGACCAGATCTTG	
Fli/6-SH R	CCA GAATTCATGTTATTGCC	
ETV1 R	TAGTAATAGCGGAGTGAACGG	19
E1AF R	GCTGGCCGGTTCTTCTGGATGC	
FEV-R	TAGCGCTTGCCATGCACCTT	
ZSG S5 R	GTCAGGAACCGAATGGGACGA	
ZSG A1 R	GCAGGGCACCTTGTCTTCATG	
EWS 7F	TCCTACAGCCAAGCTCCAAGTC	20
ERG 6R	GGTTGAGCAGCTTTCGACTG	

EWS-FLI1/ERG F	CCAAGTCAATATAGCCAACAG	18
EWS-FLI1-R	GGCCAGAATTCATGTTATTGC	
EWS/FLI1(1)-VIC	ACGGGCAGCAGAACCCTTCTTAT	
EWS/FLI1(2)-FAM	ACGGGCAGCAGAGTTCACTGCT	

3. Synovial Sarcoma

Primer name	Primer sequence (5'-3')	Reference
SYT F	AGAGGCCTTATGGATATGACCAGAT	21
SSX1/2 F	C(A/G)TTTTGTGGGCCAGATGCC	
SSX1Pr	FAM-TCCCTTCGAATCATTTTCGTCCTCTGCT	
SSX2Pr	VIC-TCTGGCACTTCCTCCGAATCATTTCCTT	
SSA F	AGACCAACACAGCCTGGACCAC	15
SSX1 R	ACACTCCCTTCGAATCATTTTCG	
SSX2 R	GCACTTCCTCCGAATCATTTC	

4. Alveolar Soft Part Sarcoma

Primer name	Primer sequence (5'-3')	Reference
ASPS-TFE3-I-F	GCCAAAGAAGTCCAAGTCGG	Not available
ASPS-TFE3-I-R	CTGTACACATCAAGCAGATTCCC	
ASPS-TFE3-II-F	CCAAAGAAGTCCAAGTCGG	
ASPS-TFE3-II-R	TGGACTCCAGGCTGATGATCTCA	
ASP F	AAAGAAGTCCAAGTCGGGCCA	22
TFE-exon 5 R	TGGACTCCAGGCTGATGATCTC	
TFE-exon 6 R	CATCAAGCAGATTCCCTGACACA	

5. Desmoplastic Small Round Cell Tumour

Primer name	Primer sequence (5'-3')	Reference
EWS-22.3 F	TCC TAC AGC CAA GCT CCA AGT C	23
WT-1 exon 9 R	GACCGGGAGAACTTTCGCTGAC	
EWS exon 8 F	GATAACCGGGGCAGGGGAATA	
EWS 7F	TCCTACAGCCAAGCTCCAAGTC	20
WT1 8R	ACCTTCGTTCACAGTCCTTG	

6. Clear Cell Sarcoma

Primer name	Primer sequence (5'-3')	Reference
EWS-CCS F1	GAGGCATGAGCAGAGGTGG	21
ATF R1	GAAGTCCCTGTACTCCATCTGTG	
EWS-CCS F3	TCCTACAGCCAAGCTCC	24
CREB R	GTACCCCATCGGTACCATTGT	
CREB-ATF R	TCCATCAGTGGTCTGTGCATACTG	
EWSR1 Ex7 F	TCCTACAGCCAAGCTCCAAGTC	
CREB1 Ex8 R	GGTATGTTTGTACGTCTCCAGAGG	21,25
EWSR1 Ex7 F	TCCTACAGCCAAGCTCCAAGTC	
CREB1 Ex7 R	GTACCCCATCGGTACCATTGT	

7. Infantile Fibrosarcoma

Primer name	Primer sequence (5'-3')	Reference
ETV6 F	AGCCCATCAACCTCTCTCAT	26
NTRK3 R	CTCGGCCAGGAAGACCTTTC	
ETV6 F	GCTTACATGAACCACATCATGG	27
NTRK3 R	GAAGTCGTGCTACAGAGAGG	

8. Myxoid Liopsarcoma

Primer name	Primer sequence (5'-3')	Reference
FUS-CHOP_I/3 F	GCAGCAGTGGTGGCTATGA	Not available
FUS-CHOP_I/3 R	TCTGCAGTTGGATCAGTCTGG	
FUS-CHOP2 F	AGCAGCAAAGCTATAATCCC	
FUS-CHOP2 R	CGAAGGAGAAAGGCAATGACT	
FUS F	CAGCCAGCAGCCTAGCTATG	
CHOP R	TGTCCCGAAGGAGAAAGGCAATG	

9. Low Grade Fibromyxoid Sarcoma

Primer name	Primer sequence (5'-3')	Reference
FUS-BBF F	CTATGGACAGCAGGACCGTGGA	Not available
FUS-BBF R	GTGAGGAGAGGGGAGCTGGAGA	
CREB3L1 R	AGTGGGGAGGTGGAGATGGCCG	
FUS F	CAGCCAGCAGCCTAGCTATG	
FUS	GCTATGAACCCAGAGGTCGT	
CREB	AGGGGCTGTGGGTCTGAG	
FP2-fc2	CAGTGGTGGCGGTTATGGCAA	28
FP3-fc2	TGGTGGTTACAACCGCAGCA	
RP1-fc2	CTGGAGGGGCTGTGGGTCTGA	
RP2-fc2	AGTTTATGAGGAGCCGTGAGG	
RP3-fc2	TCTTCTCCTCCTCTGTCAGGAC	

10. Extramyxoid Chrondrosarcoma

Primer name	Primer sequence (5'-3')	Reference
EWS-CHN_I F	GCGATGCCACAGTGTCCTATG	29
EWS-CHN_I R	ATATTGGGCTTGGACGCAGGG	
EWS-CHN_II F	CTCCAAGTCAATATAAGCCAAC	
EWS-CHN_II R	GGACGTCCGGCGAGGCGAAGC	
EWS-CHN_III F	TCTGGCAGACTTCTTTAAGCA	
EWS-CHN_III R	GGACGTCCGGCGAGGCGAAGC	
TAF2N-CHN F	GAGCAGTCAAATTATGATCAGCAGC	
TAF2N-CHN R	CCTGGAGGGGAAGGGCTAT	
EWS-7F F	CCCACTAGTTACCCACCCCA	30
EWS-12F F	GCGATGCCACAGTGTCCTATG	
TAF2N6F F	GAGCAGTCAAATTATGATCAGCAGC	
CHN 3R R	CCTGGAGGGGAAGGGCTAT	